Iron-refractory iron deficiency anemia: new molecular mechanisms

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Iron deficiency anemia is a common complication in end-stage renal disease (ESRD) and impairs the therapeutic efficacy of recombinant erythropoietin. Oral or parental iron supplements usually are effective in treating iron deficiency anemia. Some patients, however, respond poorly to iron supplements and are diagnosed as having iron-refractory iron deficiency anemia. The condition exacerbates ESRD but its underlying mechanism was unclear. Hepcidin is a central player in iron homeostasis. It downregulates the iron exporter ferroportin, thereby inhibiting iron absorption, release and recycling. In ESRD, plasma hepcidin levels are elevated, which contributes to iron deficiency in patients. Matriptase-2, a liver transmembrane serine protease, has been found to have a major role in controlling hepcidin gene expression. In mice, defects in the *Tmprss6* gene encoding matriptase-2 result in high hepcidin expression and cause severe microcytic anemia. Similarly, mutations in the human *TMPRSS6* gene have been identified in patients with iron-refractory iron deficiency. Thus, matriptase-2 is critical for iron homeostasis and may have an important role in ESRD.

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ANEMIA IN END-STAGE RENAL DISEASE

Anemia is a common complication in patients with end-stage renal disease (ESRD), as a result of progressive loss of erythropoietin (EPO) production in the kidney. Recombinant human EPO is used to treat anemia in ESRD patients. In many ESRD patients, however, high doses of recombinant human EPO fail to boost red blood cell counts to an adequate level.[1] The poor response to EPO therapy, or EPO resistance, is a major problem, which increases the morbidity and mortality in ESRD patients.[2]

Iron deficiency is a major cause of EPO resistance. Up to ~40% of patients with EPO resistance had low levels of serum ferritin or transferrin saturation.[3] As iron is an essential element in hemoglobin, when iron is in short supply, hemoglobin production is impaired. In ESRD patients, EPO resistance often reflects an underlying defect in iron homeostasis. In fact, a hemodialysis patient may lose up to 3 mg/day of iron as a result of blood loss (up to ~20 ml) during each treatment.[4] This amount of iron loss exceeds ~1 to 2 mg of daily dietary iron intake, which inevitably leads to iron store depletion and iron deficiency anemia. In comparison, peritoneal dialysis patients, who suffer much less blood loss related to the dialysis procedure, are less likely to have iron deficiency than hemodialysis patients.[5]

There are many other causes that may contribute to EPO resistance, including inflammation, dialysis procedures, hyperparathyroidism, uremia, aluminum toxicity, auto antibodies, and vitamin deficiency. These topics are covered by comprehensive reviews[1,6–8] and will not be discussed here.

Most patients with iron deficiency anemia can be treated by oral or parental iron supplements. In ESRD patients, intravenous iron supplements significantly improve the therapeutic efficacy of recombinant human EPO.[9] However, some patients with iron deficiency anemia respond poorly to iron therapy. The condition is known as iron-refractory iron deficiency anemia (IRIDA), which is characterized by a hypochromic and microcytic anemia with a low mean corpuscular erythrocyte volume, a low serum iron level, low transferrin saturation, an increased ferritin level, and a normal level of serum soluble transferrin receptor (see review[10] for laboratory diagnosis). The disease represents a

medical challenge but, for many years, its cause was unclear. Most recently, genetic studies have provided new insights into its underlying mechanisms.

IRON ABSORPTION AND BALANCE

Plasma and cellular iron levels are tightly regulated by mechanisms that control iron absorption, storage, recycling and release.[11] To absorb iron, the insoluble ferric iron (Fe^{3+}) from vegetables and grains needs to be converted into the ferrous form (Fe^{2+}) by a brush border ferric reductase in the duodenum and upper jejunum. The ferrous iron then is transferred across the enterocyte membrane by divalent metal transporter-1. The heme iron from red meat has a better bioavailability than that of the inorganic iron, and its absorption appears to be mediated by a different molecular mechanism.

In blood, iron is carried by transferrin to various tissues to be taken up by cells in a transferrin receptor-mediated endocytotic process.[11] Once inside the cell, iron is released from transferrin in the acidic endosome and stored in the form of ferritin. A significant portion, up to ~75%, of plasma iron is taken up by bone marrow to make hemoglobin in red blood precursors. Several other cell types including enterocytes, hepatocytes, and reticuloendothelial macrophages also serve as major iron storage sites.

When plasma iron levels are low, iron release is increased from enterocytes, hepatocytes, and macrophages to meet the physiological demand. Conversely, when plasma iron levels are high, iron will remain stored in these cells. The body loses iron when cells are shed from the gastrointestinal tract or blood is lost, for example, during hemodialysis. Curiously, our body lacks a way to excrete iron from the kidney, which differs from how plasma sodium levels are adjusted. As a result, regulated iron release from cellular storage sites becomes a major mechanism to control plasma iron levels.

FERROPORTIN AND HEPCIDIN IN IRON HOMEOSTASIS

The release of iron from the cell is mediated by an exporter called ferroportin,[11] a transmembrane protein on the surface of the iron-storing cells. Ferroportin exports iron from the intracellular storage pool, thereby increasing plasma iron levels. The exporter also is responsible for iron release from macrophages after erythrophagocytosis.[12] This macrophage-mediated iron recycling process provides the majority of iron supply for hemoglobin production in red blood cells.

Hepcidin is a peptide produced primarily in the liver. Its mature form consists of 25 amino acids with a calculated mass of 2.8 kDa. Hepcidin has a key role in iron homeostasis by interacting with ferroportin.[13] When it binds to ferroportin, hepcidin induces phosphorylation of the iron exporter, causing its internalization and degradation in lysosomes (Figure 1). As a result, hepcidin lowers ferroportin protein level on the cell surface, thereby inhibiting iron export from intracellular pools. Thus, hepcidin controls plasma iron level by reducing iron absorption in the gut, lowering iron release from hepatocytes, and preventing iron recycling by macrophages (Figure 1). Hepcidin expression also was detected in the kidney[14] but the biological significance of this finding is not clear.

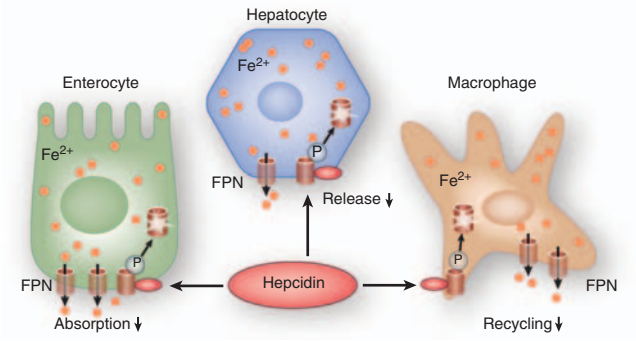

Figure 1 | Hepcidin downregulates ferroportin expression on the cell surface. Ferroportin (FPN) is expressed on the surface of enterocytes, hepatocytes, and tissue macrophages. The binding of hepcidin to FPN leads to its phosphorylation, internalization, and degradation. Low levels of FPN expression reduce iron absorption in the gut, lower iron release from the liver, and prevent iron recycling by tissue macrophages.

The importance of hepcidin in iron metabolism has been shown in genetic studies. In mice, for example, disrupting the hepcidin gene caused iron accumulation in the liver, pancreas, and heart, increased serum iron levels, and reduced iron content in the spleen.[15] Conversely, overexpression of hepcidin in transgenic mice resulted in decreased body iron levels and severe microcytic anemia.[16] Similar findings also have been reported in humans. Some patients with large hepatic adenomas had significantly high levels of hepcidin expression and decreased serum transferrin saturation.[17] The patients developed severe anemia who responded poorly to iron therapy.

HEPCIDIN IN PATIENTS WITH RENAL DISEASE

High levels of plasma or serum hepcidin have been found in patients with chronic renal disease.[18–20] In ESRD patients receiving hemodialysis or peritoneal dialysis, plasma hepcidin levels appeared to be even higher.[18,20,21] The mechanism underlying high hepcidin expression in ESRD patients is not clear but may reflect chronic inflammation in these patients. Inflammatory cytokines are strong stimuli for hepcidin expression (see below). Inflammatory monocytes and macrophages also express hepcidin, which, in an autocrine manner, reduces ferroportin expression and retains iron in these cells.[22,23] The data are consistent with the impaired iron absorption/recycling and low hemoglobin levels in ESRD patients. In addition to its role in iron metabolism, hepcidin may have a direct effect on erythropoiesis. In cell culture, hepcidin was shown to antagonize EPO-mediated erythroid colony formation, suggesting a possibility of hepcidin in inhibiting erythroid progenitor growth and/or survival.[24] This potential function of hepcidin needs to be verified in *in vivo* studies, and its significance in EPO resistance in ESRD patients remains to be determined.

HEPCIDIN GENE REGULATION

The *Hamp* gene, encoding hepcidin, is expressed primarily in the liver. Bone morphogenetic proteins (BMPs) are major activators for *Hamp* gene expression.[25,26] The binding of BMP to its cell surface receptor activates the SMAD signaling pathway and induces *Hamp* gene expression.[27] Hemojuvelin, a glycosylphosphatidylinositol-anchored membrane protein, acts as a co-receptor for BMP to promote *Hamp* gene expression. In humans and mice, mutations in the *hemojuvelin* gene reduce hepcidin expression and cause iron overload in the liver, pancreas, and heart, but reduced iron levels in tissue macrophages.[28]

In addition to BMP and hemojuvelin, other molecules also regulate hepcidin expression. For example, interleukin-6 was reported to stimulate hepcidin expression, possibly through the JAK/STAT3 signaling pathway.[29] On the other hand, growth differentiation factor-15 and hypoxia-inducible transcription factors suppress hepcidin expression.[29] *In vitro* and *in vivo* studies have shown that growth differentiation factor-15 expression was induced in response to iron depletion and in patients with iron deficiency anemia.[30] It appears that growth differentiation factor-15-mediated hepcidin inhibition is an important mechanism in regulating iron homeostasis.

The induction of hepcidin expression by inflammatory cytokines has important implications in the anemia of chronic disease.[31] Dialysis patients are known to have chronic inflammation. High levels of interleukin-6 in dialysis patients were associated with poor clinical outcomes.[32] In these patients, elevated levels of inflammatory cytokines such as interleukin-6 are expected to stimulate hepcidin expression, which will lead to reduced iron absorption in the gut and iron retention in macrophages,[33–36] thereby inhibiting erythropoiesis. Consistently, inflammatory biomarkers were linked to anemia and poor responses to EPO treatment in patients.[37,38]

MATRIPTASE-2/TMPRSS6 IN IRON METABOLISM AND ANEMIA

Matriptase-2, also called TMPRSS6, is a type II transmembrane serine protease.[39] It consists of a short cytoplasmic tail at the N terminus, a transmembrane domain and an extracellular region that includes a SEA (sea urchin sperm protein, enteropeptidase, and agrin) domain, two CUB (complement factor C1s/C1r, urchin embryonic growth factor, and BMP) domains, three low-density lipoprotein receptor repeats, and a trypsin-like protease domain at the C terminus (Figure 2). Matriptase-2 is highly expressed in the liver. Low levels of matriptase-2 mRNA were detected in the kidney, uterus, brain, adrenal gland, and testis.

Recent studies revealed a critical role of matriptase-2 in iron metabolism. In a chemically induced mutant mouse strain, *mask* characterized by extensive loss of body hair and microcytic anemia, the *Tmprss6* gene encoding matriptase-2 was found to be disrupted by a premature stop codon.[40] In these mice, dietary iron absorption was significantly reduced as a result of markedly increased hepcidin levels.

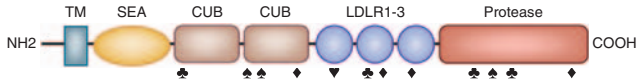

Figure 2 | Matriptase-2 domain structure and mutants identified in patients with iron-refractory iron deficiency anemia (IRIDA). Matriptase-2 consists of an N-terminal transmembrane domain (TM), one SEA (sea urchin sperm protein, enteropeptidase, and agrin) domain, two CUB (complement factor C1s/C1r, urchin embryonic growth factor, and bone morphogenetic proteins) domains, three low-density lipoprotein (LDL) receptor repeats (LDLR), and a C-terminal trypsin-like protease domain. Relative positions of nonsense (♠), missense (◆), splicing junction (♣), and frameshift (♥) mutations identified in patients with IRIDA are indicated.

Similar findings of alopecia and severe IDA were reported in *Tmprss6−/−* mice created by gene knockout.[41] *Tmprss6−/−* mice also had reduced ferroportin expression and iron accumulation in enterocytes. These studies show an important role of matriptase-2 in suppressing hepcidin expression.

Mutations in the human *TMPRSS6* gene have been found in patients with IRIDA. Several groups have identified nonsense, missense, frameshift, and splice junction mutations in the *TMPRSS6* gene in patients with familial or sporadic IRIDA[42–45] (Figure 2). In all families analyzed to date, the disease appeared to be inherited in an autosomal recessive manner. In these patients, high levels of hepcidin were present in their urine samples, consistent with the idea that *TMPRSS6* gene mutations led to higher hepcidin expression, thereby preventing iron absorption and causing IRIDA. These new findings prompted further studies to understand how matriptase-2 inhibits hepcidin expression.

Matriptase-2 is a trypsin-like enzyme.[39] A possible explanation for matriptase-2-mediated hepcidin inhibition is that the enzyme degrades hepcidin proteolytically. This hypothesis, however, was unlikely because in mice disrupting the *Tmprss6* gene increased hepcidin mRNA levels, suggesting that matriptase-2 acted at the transcriptional level.[40,41] Consistently, overexpression of matriptase-2 inhibited the *Hamp* promoter activity.[40] Most recently, matriptase-2 was shown to degrade hemojuvelin in cell membrane.[46] As hemojuvelin is a co-receptor for BMP to promote hepcidin expression, matriptase-2 seems to inhibit hepcidin expression by degrading hemojuvelin (Figure 3a). In patients and mutant mice, therefore, matriptase-2 deficiency results in elevated hemojuvelin and, subsequently, hepcidin levels. This leads to impaired iron absorption/recycling and in turn causes IRIDA (Figure 3b). Together, the latest studies have provided the first insight into the biological function of matriptase-2 and its role in iron homeostasis.

PERSPECTIVE

Iron is essential for life. Regulatory mechanisms have been evolved, allowing the body to obtain sufficient amounts of iron from the environment and yet preventing its possible

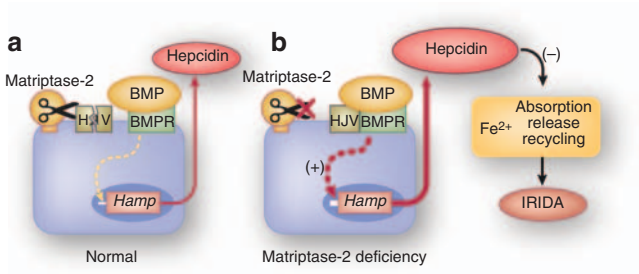

Figure 3 | Regulation of hepcidin expression by matriptase-2. Matriptase-2 prevents hepcidin overexpression by degrading hemojuvelin (HJV), which acts as a co-receptor for bone morphogenetic protein (BMP) to promote *Hamp* gene expression (**a**). In matriptase-2 deficiency (**b**), high levels of HJV enhances the BMP signaling pathway, leading to overexpression of hepcidin. Hepcidin inhibits iron absorption, release, and recycling, thereby causing IRIDA.

toxic effect of overload. Hepcidin has been identified as a central regulator in iron homeostasis. Now a critical role of matriptase-2 has been discovered in regulating hepcidin expression and in IRIDA. Membrane serine proteases are known to be involved in maintaining homeostasis. The transmembrane protease corin in the heart, for example, activates atrial natriuretic peptide, a cardiac hormone that regulates sodium homeostasis and blood pressure.[47–49] Matriptase-2 shares structural similarities with corin but instead functions in the liver to regulate iron homeostasis. It will be important to determine if additional structurally related membrane proteases are involved in regulating other metal elements that are of metabolic importance.

The finding of the matriptase-2 function in regulating hepcidin expression encourages more research to understand its role in renal disease, in which abnormal iron metabolism is common. How are matriptase-2 expression and activity regulated in ESRD patients? Is it possible that a decreased matriptase-2 activity contributes to elevated plasma hepcidin levels and exacerbates anemia in these patients? Can we take pharmacological approaches to enhance matriptase-2 expression and/or activity to improve iron absorption and prevent anemia? As iron deficiency and anemia are important complications in renal disease, studies to answer these and many other questions about matriptase-2 and hepcidin may lead to better diagnosis and treatment for ESRD patients.

DISCLOSURE

All the authors declared no competing interests.

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